



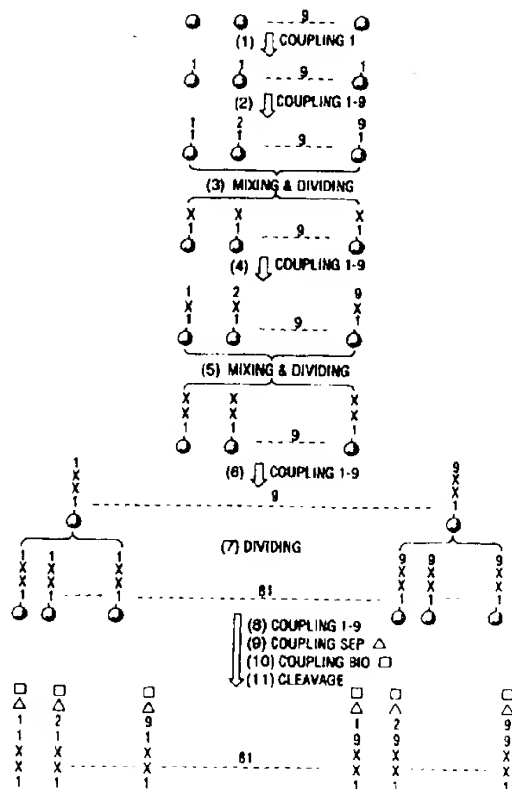
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(54) Title: AFFINITY LABELING LIBRARIES WITH TAGGED LEAVING GROUPS**(57) Abstract**

Methods and compositions are provided for identifying compounds having affinity to a target site. The method provides for the affinity group to be a leaving group from a reactive functionality capable of forming a covalent bond to the target site. One can combine the compound comprising the target site with the library, and assay for the resulting composition of the leaving groups. The leaving groups having the highest concentration can be identified as the groups having the binding highest affinity for the target site. The selected compounds may then be used for labeling the target molecule, particularly where the target molecule is naturally found in a complex mixture, such as a physiological fluid, like blood. By affinity labeling *in vivo*, the lifetime of physiologically active entities can be greatly enhanced by becoming bound to long lived blood components. The covalently bound entity may also serve as an antagonist or agonist of a particular binding protein or as an enzyme inhibitor.

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AFFINITY LABELING LIBRARIES WITH TAGGED LEAVING GROUPS

BACKGROUND OF THE INVENTION

The advent of combinatorial chemistry has provided a platform for a wide variety of opportunities. The ability to produce large libraries of different compounds means that one can screen a large array of conformations, and charge
5 distributions for their ability to bind to other compounds to act as agonists or antagonists, in binding to specific sites of a target protein, to investigate the conformation of a particular protein site, such as an enzymatic cleft or membrane channel protein, and the like.

The existence of combinatorial chemistry affords new opportunities towards
10 new therapies for diseases for which prior drugs are inadequate or for which there are no present successful therapies. The presently available drugs are inadequate for a wide variety of reasons, in many cases the pharmacokinetics of the drugs. Parenteral delivery in a bolus has many constraints on its efficacy. Since most drugs have serious side effects, one is constrained as to the upper dosage level. On the
15 other hand, drugs may undergo enzymatic modification, oxidation, degradation in the liver, secretion, and the like, so that the lifetime of the drug will be limited by the various mechanisms which serve to diminish the effective level of the drug.

Drugs which have been approved for use with humans have already been shown to be generally safe at the prescribed dosage and effective. Therefore, there
20 are substantial advantages in being able to modify these drugs, where their safety and efficacy are not unduly altered. In this way, the economies of dealing with known entities can be achieved, while at the same time increasing the available therapeutic opportunities.

BRIEF SUMMARY OF THE INVENTION

Methods and compounds are provided for directed covalent bonding of an entity to a target molecule, even site, in the presence of a plurality of chemically reactive competitive sites. Also, screening techniques are provided for identifying
5 affinity labels for directing the entity to the target molecule. The screening technique employs a library of compounds wherein the compounds have dual functions, (1) to provide non-covalent binding affinity for a specific target site and (2) to release a tagged leaving group when a chemically reactive group on the compound covalently bonds to a reactive functionality at the target site. As such,
10 one may combine a library of compounds with a target. By monitoring the rate at which the leaving groups are released, one can determine which leaving groups have the higher binding affinity for the target molecule. If one wishes to identify a particular region of the target molecule for binding, the members of the library which are assayed with the target compound may be individually screened, and the
15 preferred bonding sites for the individual members determined by analysis of the product. The identified compounds can find use in enhancing the *in vivo* lifetime of a physiologically active entity, by selectively bonding the physiologically active entity to long lived blood component target sites *in vivo*. The identified compounds may also find use for altering the activity of an enzyme, including as enzyme
20 inhibitors, for example, by providing steric hindrance at the enzyme active site, or as receptor agonists or antagonists.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a schematic illustration of the construction of an affinity labeling
25 library of the present invention.

DETAILED DESCRIPTION

In accordance with the subject invention, compounds are screened and identified for directing covalent bonding to a particular target molecule. The
30 compounds find particular use in covalently bonding an entity to a specific target molecule in the presence of a plurality of chemically reactive competitive sites on other molecules, so as to reduce the amount of non-random bonding in the mixture.

Normally, the enhancement will be at least about a factor of 1.5 over totally random bonding as determined by comparison to that bonding which occurs with a compound that exhibits no increased specificity for any particular target molecule or site over any other target molecule or site, more usually at least about a factor of 2,
5 preferably by a factor of 5, and more preferably by a factor of 10. Although bonding may be specifically directed toward a particular target molecule, rarely would one anticipate that bonding would be solely to the target molecule(s).

The compounds to be screened will, for the most part, have the following formula:



where E stands for entity; C stands for connector; S stands for separator; R stands for reactive functionality; and A stands for affinity group.

15 For the most part, the affinity group (A) will be oligomeric and, therefore, various types of affinity groups which find use include, for example, oligopeptides, oligonucleotides, oligosaccharides, combinations thereof, or the like. Generally, the affinity groups represented in any particular library will be of a common type. Oligomeric affinity groups will be characterized by being able to be readily
20 synthesized as a combinatorial library, so that the synthetic chemistry is substantially repetitive with the addition of each monomer unit to the growing oligomer. Also, methods will be available for analyzing the composition and/or sequence of the oligomeric affinity group. Alternatively, the affinity group may comprise small synthetic organic entities having a molecular weight of at least about 200, and not
25 more than about 5,000, generally ranging from about 250 to 2,000. These entities may have fluorogenic moieties or other detectable markers as attachments to assist in the detection of the affinity groups during analysis.

Generally, the oligomers employed will have at least 3 monomeric units, more usually at least 4 monomeric units, and usually fewer than 20 monomeric
30 units, more usually fewer than 12 monomeric units, preferably fewer than 10 monomeric units and more preferably in the range of about 4-8 monomeric units. With regard to the affinity groups represented in the library, one or more monomeric units of the oligomer may remain constant, thereby providing a

mechanism for reducing the overall complexity of the combinatorial library. The monomer units comprising the oligomeric affinity group may be naturally occurring or synthetic, generally being from about 2 to 30 carbon atoms, usually from about 2 to 18 carbon atoms and preferably from about 2 to 12 carbon atoms.

- 5 If the affinity group is an oligopeptide, the amino acid monomers may be naturally occurring or synthetic. Conveniently, the naturally occurring L- α -amino acids will be used, although the D-enantiomers may also be employed.

While the amino acid monomers of the oligomer may be any one of the 20 naturally occurring amino acids in either the L- or D-configuration, the amino acids
10 employed will preferentially be free of reactive functionalities, particularly reactive functionalities which would react with the reactive functionality (R) of the affinity label compound. Therefore, the amino acids which are used will usually be free of reactive amino groups, frequently also being free of thiol groups. Of particular interest are such amino acids as alanine (A), glycine (G), proline (P), valine (V),
15 serine (S), phenylalanine (F), isoleucine (I) and leucine (L) or uncharged polar amino acids like methionine (M). Other amino acids which may find use include other aromatic amino acids, such as histidine (H), tryptophan (W), and tyrosine (Y) or arginine (R), glutamate (E) and aspartate (D).

The amino acid monomers of the oligomeric affinity group may also be
20 synthetic. Thus, any unnatural or substituted amino acids of from 4 to 30, usually from 4 to 20, carbon atoms may be employed. Of particular interest are the synthetic amino acids β -alanine and γ -aminobutyrate or functional group protected amino acids such as O-methyl-substituted threonine (T), serine (S), tyrosine (Y), or the like.

- 25 Amino acids which find use may have the carboxyl group at a site other than the terminal carbon atom, may have the amino group at a site other than the α -position or may be substituted with groups other than oxy, thio, carboxy, amino or guanidino, e.g. cyano, nitro, halo, particularly fluorine, oxo, inorganic acyl groups, etc.

30 Synthetic amino acids may also be monosubstituted on nitrogen as in peptoids, which are oligomers of N-substituted glycine residues. N-substituted amino acids which find use will have an N-substituent of from about 1 to 8, usually 1 to 6

carbon atoms, which may be aliphatic, alicyclic, aromatic or heterocyclic, usually having not more than about 3 heteroatoms, which may include amino, either tertiary or quaternary, oxy, thio, and the like.

Oligopeptides may be constructed by employing standard Merrifield solid
5 phase synthetic methods, manually or by using an automated peptide synthesizer, standard protection chemistry (e.g., t-Boc or Fmoc chemistry) and resins (e.g., 4-methyl benzhydryl amine or Rink Amide resin). Successive rounds of deprotection of the terminal amino group and coupling of amino acid monomers, followed by deprotection and cleavage of peptides from resins results in the synthesis of
10 oligopeptides of the desired sequence and length. Additionally, liquid phase peptide synthesis is well known in the art and may also be employed.

If the amino acid monomers employed are N-substituted glycine residues, monomers may incorporate t-butyl-based side chain and 9-fluorenylmethoxycarbonyl α -amine protection. (See, for example, Gordon et al., J. of Medicinal Chemistry
15 (1994) 37, 1387-1385, and references cited therein). Controlled oligomerization of the N-substituted monomers may be performed manually and/or robotically with *in situ* activation by either benzotriazol-1-yloxytris (pyrrolidino)-phosphonium hexafluorophosphate or bromotris (pyrrolidino) phosphonium hexafluorophosphate. Additional steps may follow standard automated peptide synthesis protocols using α -
20 (9-fluorenylmethoxycarbonyl) amino acids.

If the affinity group is an oligonucleotide, either naturally occurring or synthetic nucleotide monomers may be employed. Particularly, for synthetic nucleotides, the phosphate or sugar groups may be modified where phosphate may be substituted by having the oxygen atoms replaced with sulfur or nitrogen, the
25 phosphate group may be replaced with sulfonate, amide etc., the ribose or deoxyribose may be replaced with 5 to 6 carbon atom sugars such as arabinose, fructose, glucose, or the like, and the purines and pyrimidines may be modified by substitution on nitrogen, with alkyl or acyl, may employ different ring structures, may have nitrogen replaced by oxygen, or vice versa, and the like.

30 If the affinity group is an oligosaccharide, the oligosaccharide will usually have from 4 to 6 monomeric units which may be linear or branched, comprised of sugars of from 5 to 8 carbon atoms. Various modifications of known

oligosaccharides may be employed, particularly where one is interested in binding to lectins or adhesion molecules.

Combinatorial libraries of affinity groups may be prepared in accordance with conventional ways for producing combinatorial libraries, particularly using a solid support and adding the monomeric components in a stepwise manner. See, for example, U.S. Patent Nos. 4,883,092; 5,010,175; 5,182,366 and 5,270,170 and PCT application Nos. WO92/00091; WO92/09300; WO93/06121; WO93/20242; WO94/06451; WO94/06291 and WO95/28640, as exemplary of a much larger literature of techniques. Preferably, the synthetic chemistry is substantially repetitive with the addition of each monomer unit to the growing oligomer.

Initially, one will have a combinatorial library of compounds having varying affinity groups which are to be screened for their affinity to a target molecule or site. Generally, the library will have compounds representing at least 50 different affinity groups, frequently 100 different affinity groups, usually at least about 500 different affinity groups, preferably at least about 1000 different affinity groups, and may be 10,000 or more different affinity groups, although usually the libraries will have 5,000 or fewer different affinity groups. The library may have greater proportions of one compound over other compounds, but desirably the relative concentrations will differ by less than about 50%, preferably less than about 25%. For the screening process, the libraries may be divided into smaller units, which will generally range from about 5 to 1,000, frequently from about 5 to 500, usually from about 10 to 500 moieties and more usually from about 10 to 250. The source of the affinity groups may be any convenient source including combinatorial libraries, natural products, stored synthetic compounds, and the like.

The entity (E) may be any of a wide variety of biologically active or non-biologically active compounds which find application for bonding to targets in a complex mixture. Entities are such that they perform the desired function in the environment in which they become covalently bonded. For the most part, that environment will be an aqueous environment, usually serum or the interstitium. For use as drugs, the entity may be an agonist, an antagonist, a specific binding compound, an enzyme inhibitor (where the enzyme may be either soluble or membrane bound), a metal chelator, a factor supplement, a molecular scavenger,

such as vitamin E, or the like. More specifically, the entity may include thrombin inhibitors, such as argatroban, renin inhibitors, ACE inhibitors, inhibitors of the coagulation and complement cascade and serine proteases, $\alpha_2\beta_2$ antagonists, GPIIb/IIIa antagonists, CRF antagonists, or the like.

5 Thus, the nature of the entity may be varied widely, depending upon its purpose. In one application, the entity may serve as a physiologically active compound, where the purpose of the subject compound is to bond the physiologically active compound to a long lived blood component. The affinity label serves to enhance the amount of the entity which is bound to a particular target
10 site. For the most part, when used as a physiologically active compound, the subject compound will be combined with a complex mixture, such as blood, cells, tissue, or the like. Particularly, the subject compounds may be administered *in vivo* as described in co-pending application Serial No. 08/237,346, whose disclosure is incorporated herein by reference. When used with blood, the primary targets will
15 be immunoglobulins, red blood cells, particularly glycoprotein proteins of the red blood cells, serum albumin, and platelets, however, other target sites are available.

 In another application, the entity may be a compound which allows the diagnostic visualization of specific sites or compartments within the body by employing such diagnostic techniques as positron emission tomography (PET),
20 computerized tomography (CT), single photon emission computerized tomography (SPECT), magnetic resonance imaging (MRI), nuclear magnetic imaging (NMI), fluoroscopy, ultrasound, etc. For such applications, the entity may comprise such things as contrast agents, radioisotopes of such elements as iodine (I), including ^{123}I , ^{125}I , ^{131}I , etc., barium (Ba), gadolinium (Gd), technetium (Tc), including ^{99}Tc ,
25 phosphorus (P), including ^{31}P , iron (Fe), manganese (Mn), thallium (Tl), chromium (Cr), including ^{51}Cr , carbon (C), including ^{11}C , or the like, fluorescently labeled compounds, etc. Such entities are also useful for identifying the presence of particular target sites in a mixture, to label molecules in a mixture, and the like.

 In yet another application, the entity may be chosen such that it functions to
30 sterically hinder or alter the binding specificity of a specific target binding protein. Such entities may take many forms which are readily determinable by those skilled in the art, and include various chemical groups, including acetyl, methyl, ethyl,

propyl, and the like. Such entities may also comprise amino acids, oligomeric structures which themselves serve to provide binding affinity for the target molecule, much like the affinity groups described *supra* or small molecules such as biotin, or the like. Such entities find use, for example, for inhibiting the ability of a binding
5 protein to bind to its substrate and for inactivating cell membrane proteins, such as ion conducting channels, enzymatic proteins, specific receptors, and the like. Such entities may also serve a research purpose, for example, in allowing the dissection of the function of a variety of surface membrane proteins or for the identification and/or purification of the labeled target molecule.

10 In each of the above applications, covalent bonding of the entity serves to affect the biological function of the target molecule to which it is bound. Thus, by employing the subject compositions, one may modify the nature of the target molecule, change the characteristics of the target molecule, allow for the identification and/or isolation of the target molecule, etc.

15 The affinity group (A) will be bonded to the entity (E) through a unit, usually a multicomponent unit, where the unit will comprise a chemically reactive functionality which will react with a reactive functionality at the target site to form a covalent bond. Covalent bond formation at the target site, in turn, results in covalently bonding the entity to the target site with concomitant liberation of the
20 affinity group.

The reactive functionality, R, will generally be stable in an aqueous environment and will usually be carboxy, phosphoryl, or convenient acyl group, either as an ester or a mixed anhydride, or an imidate, thereby capable of forming a covalent bond with an amino group at the target site to form an amide or amide
25 derivative. For the most part, the esters will involve phenolic compounds, or be thiol esters, alkyl esters, phosphate esters, or the like.

While the reactive functionality (R) is usually chosen to react with an amino group at the target site, other functionalities at the target site may be exploited. For example, the reactive functionality (R) may comprise various phosphinyl or
30 phosphonyl derivatives for the bonding to available hydroxyl functions at the target site or may comprise an imine, thioimine or disulfide for bonding to thiol residues.

The reactive functionality may also comprise any of a number of well known chemically reactive alkylating agents.

The a and b of the formula presented above will have a numerical value so that $a + b = 1$. Therefore, upon covalent bonding of the entity through the reactive
5 functionality to the target site, the affinity group will be liberated.

The separating component (S) of the intermediate unit between the entity (E) and the affinity group (A) may be a bond or may be present to provide various functions. The separator may provide for synthetic convenience, particular physical characteristics of the total composition, e.g. water solubility, reduced non-specific
10 binding, the group bonded to the reactive functionality to provide an active ester, mixed anhydride, imidate, phosphate derivative, imine, thioimine, or the like. For the most part, the separating component, when other than a bond, will have from about 2-30 carbon atoms, and from about 0-10, more usually 1-8 heteroatoms, which for the most part will be O, N, P, S, and halogen. The particular separating
15 group may not be critical to this invention, as indicated being selected based on the nature of the entity and the affinity group to provide convenient characteristics for the molecule. Of particular interest, where the separator component is involved with the reactive functionality R, is to have an aromatic carboxylic acid derivative, so that the heteroatom of the reactive functionality is bonded directly to an aromatic
20 carbon atom. Alternatively, one may have a carboxysuccinimidyl, sulfosuccinimidyl, or thiosuccinimidyl, for insertion as the separating group. As illustrative are *p*-thiolphenylacetic acid, *o*-, *p*- and *m*-tyrosine, *m*-nitrotyrosine, *p*-thiolphenylalanine, *o*-, *m*- or *p*-thiobenzoic acid, and the like, i.e., units to be employed in the reactive functionality/separator complex.

25 The next component of the affinity label compound is the connector, C. The connector will be selected in a manner similar to the separator. Thus, the connector may have various functions in relation to the entity, in enhancing the availability of the entity by providing for enhanced water solubility in relation to the entity, by providing for a useful linking group between the entity and the adjacent unit, by
30 enhancing the desired activity of the entity, for providing for the manifestation of the entity's desired function, or the like. For the most part, the connector will be bifunctional of from about 1-20 atoms in the chain, which atoms may be carbon,

nitrogen, oxygen, sulfur, phosphorus, and the like. The connector may be alkylene groups, generally of from 2-16, more usually of from 2-8 carbon atoms, polyoxyalkylene groups, where the alkylene groups will be of 2-3 carbon atoms, and having from 1-8, more usually of from about 1-6 units, an amino acid, including
5 alpha and omega amino acids, or oligopeptide having from 1-8, usually 1-6 amino acids, where the amino acids may be polar or non-polar, charged or uncharged, aliphatic, alicyclic, aromatic or heterocyclic, naturally occurring or synthetic. The connector may also be have the structure of an affinity group as described above, thereby providing additional binding affinity at the target site.

10 As is evident from the above, the various units of the affinity label compound will be selected in relation to the nature of the entity and the affinity group, particularly the entity. Thus, the components of the multicomponent unit will be selected in relation to the use of the compound, and the effect of the components on the intended use of the compound.

15 For identification of affinity label members of the library having increased affinity for a particular target site relative to other members of the library, as indicated previously, depending upon the size of the library, all or a portion of the members of the library may be combined with the pure target compound in an appropriate reaction medium. The medium will vary widely, depending upon the
20 nature of the target compound, and the environment in which the subject affinity label compounds will be used for bonding to the target compound. For the most part, the media will be polar, particularly aqueous, and may be buffered or otherwise modified to as to more closely mimic the ultimate environment in which the subject compounds will be used. The concentrations of the target compound and
25 the library members may be varied widely, usually being determined empirically, so as to optimize the differentiation between the various members of the library. Generally, for screening purposes, concentrations of the target compound will be in the range of about 0.05 to 5 μM , preferably in the range of about 0.1 to 1.0 μM , while concentrations of the library will vary in the range of about 10 to 150 μM ,
30 preferably in the range of from about 50 to 100 μM and more preferably in the range of from about 75 to 85 μM .

The temperature of the reaction may be varied over a broad range compatible with the stability of the components of the system, frequently being room temperature or the temperature of the environment in which the subject compounds will be used. To the extent that the subject compositions will be used

5 physiologically, the temperature will generally be in the range of about 34-40°C, more usually about 37°C.

The determination of affinity for the target exhibited by the various affinity label members of the library may be made by determining the composition of the liberated affinity groups at a single time point or a plurality of time points after the

10 reaction is initiated. For the most part, those affinity groups which are liberated the earliest after the reaction is initiated are those which exhibit the greater binding affinity for that target site. Usually, the reaction will be allowed to proceed until there is a sufficient population of liberated affinity groups to allow for their ready determination and differentiation. The affinity label leaving groups may be analyzed

15 by any convenient means, including mass spectrometry, gel electrophoresis, chromatography, e.g. hplc, tlc, or the like, where if appropriate, the separated components may then be sequenced. Where a plurality of aliquots of the library used, those sequences demonstrating preferred affinities may then be combined in a subsequent determination for direct comparison.

20 The subject affinity label compounds will be synthesized in accordance with conventional ways. Synthesizers are commercially available for synthesizing oligonucleotides and oligopeptides. See the references cited above. Various conventional chemistries may be employed. Depending upon the nature of the functional group, the separator (S), the connector (C), and the entity (E) synthetic

25 strategies will be devised which allow for synthesis of the molecule at reasonable yields, and without the formation of complex mixtures. The particular synthetic strategy will be determined empirically, and on a case by case basis. Methods for combining various compounds are well known in the literature, and can be employed with advantage. Where precursors are known to the entity, particularly prodrugs for

30 drugs, the prodrugs will frequently indicate the site for attachment and the nature of a linking group. Where prodrugs are not available, the physiologically active

molecule may be modified stepwise at different sites, and the activity of the resulting compound determined.

When administered physiologically, the targets will usually be proteins, either individually, as aggregates with the same or different proteins or as surface membrane proteins. Target proteins may be long lived in that they have a half life of at least about 12 hours, preferably at least about 48 hours, more preferably at least about 5 days. Particular proteins, either individually or as part of cells, include the surface membrane proteins of erythrocytes, particularly glycophorin A and Band 3, and C, T or B cell surface proteins, such as CD3, B7, p28, CTLA-4, CD34, Thy1, CD4, CD8, LFA1, CD5, sIgE, sIgM, platelet proteins, such as IIb/IIIa, leukocyte surface membrane proteins, serum albumin, immunoglobulins, particularly IgG and IgM, apolipoproteins, such as LDL, HDL and VLDL, and proteins associated with chylomicrons, endothelial cell surface proteins, such as integrins, adhesion proteins, etc. Cells for targeting include platelets, erythrocytes, endothelial cells, T cells or subsets thereof, B cells or subsets thereof, other leukocytes, such as macrophages, monocytes, neutrophils, basophils, NK cells, eosinophils, stem cells, such as hematopoietic stem cells, tumorous or malignant cells, infected cells, such as virally infected, e.g., retroviruses such as HIV, DNA viruses, such as hepatitis B or C virus, etc. The cells may be fixed or mobile.

A number of proteins of interest have numerous reactive functionalities for reaction *ex vivo* or *in vivo*. For example, human serum albumin, glycophorins, thrombin, adenylate kinase, plasminogen, β -lactamase, ACE, glutathione transferase, HMG CoA reductase, gastric lipase, and lecithin:cholesterol acyl transferase have active amino groups for conjugation. Cathepsin cysteine proteases and cytosolic phospholipase A₂ have numerous thiol groups for conjugation.

The subject affinity label compounds, when administered physiologically, will usually be administered as a bolus, but may be introduced slowly over time by transfusion using metered flow, or the like. Alternatively, although less preferable, blood may be removed from the host, contacted with the affinity label compound *ex vivo*, and returned to the host. The affinity label compounds will be administered in a physiologically acceptable medium, e.g., deionized water, phosphate buffered saline, saline, mannitol, aqueous glucose, alcohol, vegetable oil, or the like.

Usually, a single injection will be employed although more than one injection may be used, if desired. The affinity label compounds may be administered by any convenient means, including syringe, catheter, or the like. The particular manner of administration will vary depending upon the amount to be administered, whether a
5 single bolus, sequential, or continuous administration, or the like. Administration will be intravascular, where the site of introduction is not critical to this invention, preferably at a site where there is rapid blood flow, e.g., intravenously, peripheral or central vein. The intent is that the compound administered be effectively distributed in the vascular system so as to be able to react with target molecules
10 therein.

The dosage of the affinity label compound will depend upon the entity being employed and will, therefore, be dependent on the adverse effects of the entity of interest, if any, the time necessary to reduce the unbound concentration of the affinity label compound present in the vascular system, the indication being sought,
15 the sensitivity of the compound to destruction by vascular components, the route of administration, and the like. As necessary, the dosage of affinity label compound may be determined empirically, initially using a small multiple of the dosage normally administered, and as greater experience is obtained, enhancing the dosage. Dosages will generally be in the range of 1 ng/Kg to 10 mg/Kg, usually being
20 determined empirically in accordance with known ways, as provided for in preclinical and clinical studies.

The following examples are offered by way of illustration, and not by way of limitation.

25

EXPERIMENTAL

A. Construction of p-Thiobenzoic-acid Containing Affinity Labeling Libraries

An affinity labeling library with tagged leaving groups and having a p-thiobenzoic acid residue as a separator was designed and constructed. The structure of the affinity labeling library members is described as follow:

30

Biotin-SPhCO-O₁-Q₂-X₁-X₂-B-NH₂ (81 wells, 81 compounds/well)

In the above described structure, X_1 and X_2 represent that all of the 9 selected L-amino acids appear in a well, O_1 represents that only one of the 9 selected L-amino acids appear in a well, Q_2 represents that only one of the 9 selected D-amino acids appears in a well, and B represents that only one of the 9 selected L-amino acids appears in all 81 wells of a set. Biotin serves as the "entity" which becomes covalently bound to the target protein. "SPhCO" represents the S-fluorenylmethyl-p-thiobenzoic acid-derived separator between the biotin "entity" and the oligomeric tagged leaving group.

The 9 amino acids employed were carefully selected by considerations of the side chain functional groups and are listed in Table 1.

TABLE 1

Code	Amino Acid	Category	Starting material
15 1	glutamic acid	acid	Fmoc-Glu(OtBu)-OH
2	glutamine	amide	Fmoc-Gln(Trt)-OH
3	arginine	base	Fmoc-Arg(Pmc)-OH
4	methionine	sulfide	Fmoc-Met-OH
5	serine	alcohol	Fmoc-Ser(tBu)-OH
20 6	tyrosine	phenol	Fmoc-Tyr(tBu)-OH
7	leucine	aliphatic	Fmoc-Leu-OH
8	phenylalanine	aromatic	Fmoc-Phe-OH
9	tryptophan	aromatic	Fmoc-Trp(Boc)-OH

The D-amino acids, Q_2 were introduced in order to prevent facile peptidyl cleavage *in vivo* and to maximize the affinity to the macromolecular targets.

In each set there are 81 wells and in each well there are 81 compounds, a proper size for assay, while B is fixed. Such results in a library of 6561 total library members.

For example, when glutamic acid (E) was chosen to be the residue "B" in the above formula, the set 1 of combinatorial p-thiobenzoic-containing affinity labeling libraries Biotin-SPhCO- O_1 - Q_2 - X_1 - X_2 -E-NH₂ was constructed as follows:

The synthesis utilized Merrifield solid phase peptide synthesis methods and Fmoc chemistry. Rink amide resin was used to furnish amide at the C-terminus.

A schematic illustration outlining the construction of this set of libraries described above is shown in Figure 1.

- 5 In step (1) shown in Figure 1, Fmoc-Glu(OtBu)-OH was coupled to an identical amount of resin support in all 9 reaction vessels after removal of the Fmoc protecting group on Rink amide resin.

- Removal of the Fmoc group was performed by treating with 20% piperidine in NMP twice, 2 minutes and 15 minutes, respectively at room temperature,
10 followed by several NMP washes.

- Coupling of the Fmoc-Glu(OtBu)-OH to the resin support was performed by adding to the resin in a reaction vessel with an N,N-dimethylformamide (DMF) solution of the amino acid (4 equivalents), O-benzotriazol-1-yl-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU) (4 equivalents), 1-
15 hydroxbenzotriazole (HOBt) (4 equivalents) and diisopropylethyl amine (DIEA) (8 equivalents). The reactions were allowed to proceed for 2 hours at room temperature. Completion of each reaction was monitored by a ninhydrin test. The coupling reactions were repeated until the ninhydrin test for each reaction was negative. Once coupling was complete as evidenced by a negative ninhydrin test,
20 the peptide resin in each of the nine separate reactions was washed with N,N-dimethyl formamide (DMF).

- Before the second L-amino acids were coupled to an identical amount of resin bearing the same Fmoc protected amino acid (i.e., Fmoc-Glu(OtBu)-OH) in all 9 reaction vessels), the Fmoc protecting group was removed by treating with 20%
25 piperidine in NMP twice, 2 minutes and 15 minutes, respectively, at room temperature, followed by several NMP washes.

- As shown in step (2) of Figure 1, each of the nine different Fmoc-protected L-amino acids shown in Table 1 were then individually linked to an identical amount of resin in all 9 reaction vessels. Coupling reactions were again carried out by
30 adding to the resin in a reaction vessel with an N,N-dimethyl formamide (DMF) solution of the amino acid (4 equivalents), O-benzotriazol-1-yl-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU) (4 equivalents), 1-

hydroxybenzotriazole (HOBt) (4 equivalents) and diisopropylethyl amine (DIEA) (8 equivalents). The reactions were allowed to proceed for 2 hours at room temperature. Completion of each reaction was monitored by a ninhydrin test. The coupling reactions were repeated until the ninhydrin test for each reaction was
5 negative. Once coupling was complete as evidenced by negative ninhydrin test, the peptidyl resin in each of the nine separate reactions was washed with N,N-dimethyl formamide (DMF).

The result of this second round of coupling is the generation of nine equimolar independent and distinct pools of dipeptides linked to resin support. As
10 shown in Figure 1, since each of the nine independent and distinct pools contains one distinct dipeptide, there are now generated a total of 9 distinct dipeptides.

As shown in step (3) of figure 1, the resins from each of the nine independent and distinct pools generated in step (2) were mixed and then divided into identical pools. This gives rise to nine identical pools each consisting of nine
15 distinct dipeptides linked to the resin support.

Before the third round of coupling, the Fmoc protecting group was removed by treating with 20% piperidine in NMP twice, 2 minutes and 15 minutes, respectively, at room temperature, followed by several NMP washes.

As shown in step (4) of Figure 1, each of the nine different Fmoc-protected
20 L-amino acids shown in Table 1 were individually linked to an identical pool of resin in all 9 reaction vessels. Coupling reactions were carried out as described above.

As shown in step (5) of figure 1, the resins from each of the nine independent and distinct pools generated in step (4) were mixed and then divided
25 into identical pools. This gives rise to nine identical pools each consisting of eighty one distinct tripeptides linked to the resin support.

Before the fourth round of coupling, the Fmoc protecting group was removed by treating with 20% piperidine in NMP twice, 2 minutes and 15 minutes, respectively at room temperature, followed by several NMP washes.

30 As shown in step (6) of Figure 1, each of the nine different Fmoc-protected amino acids shown in Table 1 were identically linked to an identical pool of resin in all 9 reaction vessels. In this round of coupling, unlike all of the other rounds of

coupling, amino acids in the D-configuration are employed. Coupling reactions were again carried out as described above.

The results of this fourth round of coupling is the generation of nine equimolar independent and distinct pools of tetrapeptide linked to resin support. As shown in Figure 1, since each of the nine independent and distinct pools contains eighty one distinct tetrapeptides, there are now generated a total of 729 distinct tetrapeptides.

As shown in step (7) of Figure 1, each of the nine independent and distinct pools of tetrapeptides generated in step (6) were each divided in to a set of nine identical pools without mixing the pools before dividing. Division gives rise to nine distinct sets each containing 9 identical units (81 pools total, each containing 81 tetrapeptides). The step of splitting the pools without mixing allows for the identity of the fourth and fifth amino acids coupled to the resin to be defined. Before the fifth round of coupling, the Fmoc protecting group was removed by treating with 20% piperidine in NMP twice, 2 minutes and 15 minutes, respectively, at room temperature, followed by several NMP washes.

As shown in step (8) of Figure 1, each of the nine identical pools in each of the nine sets of pools generated in step (7) was then coupled to one of the nine Fmoc-protected amino acids shown in Table 1. Coupling reactions were again carried out as described above.

The results of this fifth round of coupling is the generation of eighty one equimolar independent and distinct pools of pentapeptides linked to a resin support. As shown in Figure 1, since each of eighty one independent and distinct pools contains eighty one distinct pentapeptides, there are now generated a total of 6561 distinct pentapeptides.

As shown in step (9) of Figure 1, each of the eighty one independent and distinct pools generated in step (8) was then coupled to a common protected separator S-fluorenylmethyl-p-thiobenzoic acid. Coupling reactions were carried out by adding to the resin in a reaction vessel with a N,N-dimethyl formamide (DMF) solution of S-fluorenylmethyl-p-thiobenzoic acid (4 equivalents), O-benzotriazol-1-yl-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU) (4 equivalents), 1-hydroxybenzotriazole (HOBt) (4 equivalents) and diisopropylethyl amine (DIEA) (8

equivalents). The reactions were allowed to proceed for 2 hours at room temperature. Completion of each reaction was monitored by a ninhydrin test. The coupling reactions were repeated until the ninhydrine test fore each reaction was negative. Once coupling was complete as evidenced by a negative ninhydrin test,
5 the peptidyl resin in each of the nine sepearate reactions was washed with N,N-dimethyl formamide (DMF).

Before the last round of coupling, the fluorenylmethyl protecting group was removed by treating with 20% piperidine in NMP twice, 2 minutes and 10 minutes, respectively at room temperature, followed by NMP washes rapidly.

10 As shown in step (10) of Figure 1, each of eighty one independent and distinct pools generated in step (8) was then coupled to biotin. Coupling reaction were carried out by adding to the resin in a reaction vessel with a N,N-dimethyl formamide (DMF) solution of biotin (5 equivalents), O-benzotriazol-1-yl-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU) (5 equivalents), 1-
15 hydroxybenzotriazole (HOBt) (5 equivalents) and diisopropylethyl amine (DIEA) (10 equivalents). The reactions were allowed to proceed for 2 hours at room temperature. The coupling reactions were repeated to ensure completion.

Finally, to complete the construction of the p-thiobenzoic-containing affinity labeling libraries, as shown in step (11) of Figure 1, the completely constructed
20 affinity labeling libraries were cleaved from the resin component and purified. The cleavage and purification were performed as follows.

The peptidyl resin mixtures in each 81 independent and distinct pools generated in step (10) were dried. Each was then independently treated with trifluoroacetic acid (TFA)/H₂O (95/5, v/v) for 1.5 hours. The peptide/TFA solution
25 was then extracted twice with ether. Following ether extraction, the peptide solution was lyophilized giving rise to the completely constructed and dried p-thiobenzoic-containing affinity labeling libraries.

The final combinatorial p-thiobenzoic-containing affinity labeling libraries are substantially stable under normal storage conditions as determined by HPLC
30 (high performance liquid chromatography) mass spectral analysis.

B. Screening of Affinity Labeling Library

The screening of libraries that contain an entity which becomes covalently bonded to the target protein as a result of the affinity labeling reaction may be conducted in two sequential steps and serves as a specific example of a general method for detecting the existence of a covalent adduct. The first step of the

5 screening process is the reaction of a given target protein with the affinity labeling library or portion thereof. These reactions are typically conducted at room temperature, although not exclusively so, in the wells of polypropylene 96-well plates. Specified wells contain the target protein that is typically present at a concentration between 0.1 and 1 μ M in a buffered solution that is of an appropriate

10 composition and pH for maintaining the native biological structure and function of the target protein. Labeling reactions are initiated by the addition of a solution of affinity labeling library members in DMSO to give typical concentrations of total library and DMSO of 80 μ M and 2% (volume/volume), respectively. The labeling reactions are terminated at a specified time, which may be either as short as 5

15 seconds or longer than 1 hour, by the addition of a suitable quench reagent (i.e. hydroxylamine for quenching thioesters) that combines with unreacted library members to give forms of the library that no longer react with the target protein. Specified wells of the plate contain reaction mixtures that measure the amount of non-covalent binding of the entity to the target protein. These control reactions are

20 conducted by first mixing the library with the quench reagent for a time sufficient to give forms of the library that do not react with the target protein and then subsequently adding the target protein.

The detection of the entity that is covalently bound to the target protein is the second step of the screen and uses standard methodologies which are known in the

25 art. For example, if the entity that becomes covalently bonded to the target protein is biotin, Enzyme-Linked-Immunoassays (ELISA's) can be employed to detect covalent bonding. Briefly, polystyrene 96-well plates can be coated with an antibody that specifically binds the target protein and modified forms thereof. A portion of each reaction mixture may then be transferred to the corresponding wells

30 of the antibody-coated plate, and the binding of target protein to antibody allowed to ensue for approximately 2 hours. The plate is then emptied and 10 times filled with and emptied of phosphate buffered saline (PBS, 10 mM Pi, pH 7.4, 137 mM NaCl,

2.7 mM KCl). This washing procedure removes non-covalently bound biotin from the well. A solution containing the enzyme conjugate avidin-horse-radish peroxidase may then be added to the washed well, and the binding of avidin of the conjugate to biotin of the modified target molecule, which is bound to the antibody of the plate, is complete after 30 minutes. The plate may then be washed as described above, and hydrogen peroxide and orthophenyl diamine are added as substrates of the peroxidase enzyme to give a visual measure of the amount of conjugate present in each well; conjugate amount is proportional to the amount of modified target protein in the well. The optical densities of each well are measured, and the values obtained are recorded in a computer spreadsheet and analyzed.

While the ELISA method of screening that is described above is specific for biotin-containing proteins, the method is, in principle, applicable for measurement of any Affinity Labeling Library that results in the formation of a detectable adduct with any target biomolecule that can be sequestered at the surface of a 96-well plate.

Mass spectrometry may be used to determine the chemical composition of the pentapeptide affinity groups that are released when a library member(s) reacts with a given target protein. Each released affinity group will have a unique molecular mass that can be measured following liquid chromatography; the measured mass(es) will identify the affinity group(s) of the members of the library that has reacted with the target protein. For example, when the protease of the HIV virus is reacted with two mixtures of nine library members (either of composition Biotin-SPh-CO-Arg-Arg-Glu-O-Glu-NH₂ or Biotin-SPh-CO-Arg-Arg-Met-O-Glu-NH₂, where O is one of nine different amino acids), the formation of biotinylated protease is detected in each reaction. Released affinity groups, where position O is either Met, Tyr and Leu or Ser, Gln, Arg and Tyr, are detected for the library members Biotin-SPh-CO-Arg-Arg-Glu-O-Glu-NH₂ and Biotin-SPh-CO-Arg-Arg-Met-O-Glu-NH₂, respectively.

It is evident from the above, that the subject invention provides an efficient approach to identifying affinity label compounds which can be used to direct a specific entity to a target site. Thus, the subject invention allows for marking specific targets, particularly proteins, for a wide variety of purposes. Where complex mixtures are involved, such as blood, one can provide for enhanced bonding to a specific component in the blood, more particularly, a specific site on

the target. In this way, one may provide for specific targeting to mobile components, such as blood cells, e.g. erythrocytes and platelets, proteins, such as immunoglobulins, serum albumin, transferrin, and the like. In mixtures in culture, one may specifically inhibit an enzyme target, so as to prevent enzymatic

5 interference with the culture. For example, one may provide for specific bonding to RNases to prevent degradation of RNA. One may inhibit specific hydrolases, oxidoreductases, or the like. Where one wishes to mark a particular target, one can provide for fluorescent, radioactive, or other entity, which may be detected.

All publications and patent applications mentioned in this specification are
10 herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto
15 without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for determining an affinity group sequence which provides for an enhanced level of covalent bonding of an entity of interest to a target molecule, said method employing a library of affinity label molecules, where said library comprises
5 a plurality of potential affinity groups joined, through a reactive functionality capable of covalent bonding to said target molecule and having said affinity group as a leaving group, to said entity of interest, said method comprising:
combining at least a portion of said library with said target molecule under reaction conditions, whereby said affinity label molecules react with said target
10 molecule in proportion with the affinity of said affinity group for said target molecule; and
analyzing the mixture of affinity groups free of said entity of interest to determine the relative affinity of said affinity groups with said target site.
- 15 2. A method according to Claim 1, wherein said at least a portion comprises at least 50 different affinity groups.
3. A method according to Claim 1, wherein said affinity group is an oligopeptide.
20
4. A method according to Claim 1, wherein said affinity group is an oligonucleotide.
5. A method according to Claim 1, wherein said affinity group is an
25 oligosaccharide.
6. A method according to Claim 1, wherein said entity of interest is a drug.
7. A method according to Claim 1, wherein said target molecule is a long lived
30 blood component.

8. A method according to Claim 7, wherein said long lived blood component is an erythrocyte or platelet.
9. A method according to Claim 7, wherein said long lived blood component is serum albumin or immunoglobulin.
10. A method according to Claim 1, wherein said target molecule is an enzyme, a cell membrane channel or a receptor.
11. A method for determining an affinity group sequence which provides for an enhanced level of covalent bonding of an entity of interest to a target molecule. said method employing a library of affinity label molecules, where said library comprises a plurality of potential affinity groups joined, through an ester reactive functionality capable of covalent bonding to said target molecule in an aqueous medium and having said affinity group as a leaving group, to said entity of interest, said method comprising:
- combining at least a portion of said library with said target molecule under reaction conditions, whereby said affinity label molecules react with said target molecules in proportion with the affinity of said affinity group for said target molecule; and
- analyzing the mixture of affinity groups free of said entity of interest by means of mass spectrometry to determine the relative affinity of said affinity groups with said target site.
12. A method according to Claim 11, wherein said affinity group is an oligopeptide.
13. A method according to Claim 11, wherein said affinity group is an oligonucleotide.
14. A method according to Claim 11, wherein said affinity group is an oligosaccharide.

15. A method according to Claim 11, wherein said entity of interest is a drug.
16. A method according to Claim 11, wherein said target molecule is a long lived blood component.
- 5 17. A method according to Claim 16, wherein said long lived blood component is an erythrocyte or platelet.
18. A method according to Claim 16, wherein said long lived blood component
10 is serum albumin or immunoglobulin.
19. A method for altering the activity of an enzyme, said method comprising:
identifying an affinity label molecule having an affinity group sequence
which provides for an enhanced level of covalent bonding of an entity of interest to
15 said enzyme, and
contacting said enzyme with said affinity label molecule such that said entity
of interest becomes covalently bonded to said enzyme,
wherein the presence of the covalently bonded entity of interest on said
enzyme functions to alter the activity of said enzyme.
- 20 20. A method for antagonizing a receptor molecule, said method comprising:
identifying an affinity label molecule having an affinity group sequence
which provides for an enhanced level of covalent bonding of an entity of interest to
said receptor molecule, and
25 contacting said receptor molecule with said affinity label molecule such that
said entity of interest becomes covalently bonded to said receptor molecule,
wherein the presence of the covalently bonded entity of interest on said
receptor molecule functions to antagonize said receptor molecule.
- 30 21. A composition comprising at least 50 different affinity label molecules, each
of said affinity label molecules comprising different oligomeric affinity groups
joined to an entity of interest through a reactive functionality capable of forming a

covalent bond with a target molecule with said oligomeric affinity groups being the leaving groups.

22. A composition according to Claim 21, wherein said reactive functionality is
5 an ester capable of reacting with an amino group in an aqueous medium.
23. A composition according to Claim 21, wherein said oligomeric affinity group is an oligopeptide, an oligonucleotide or an oligosaccharide.

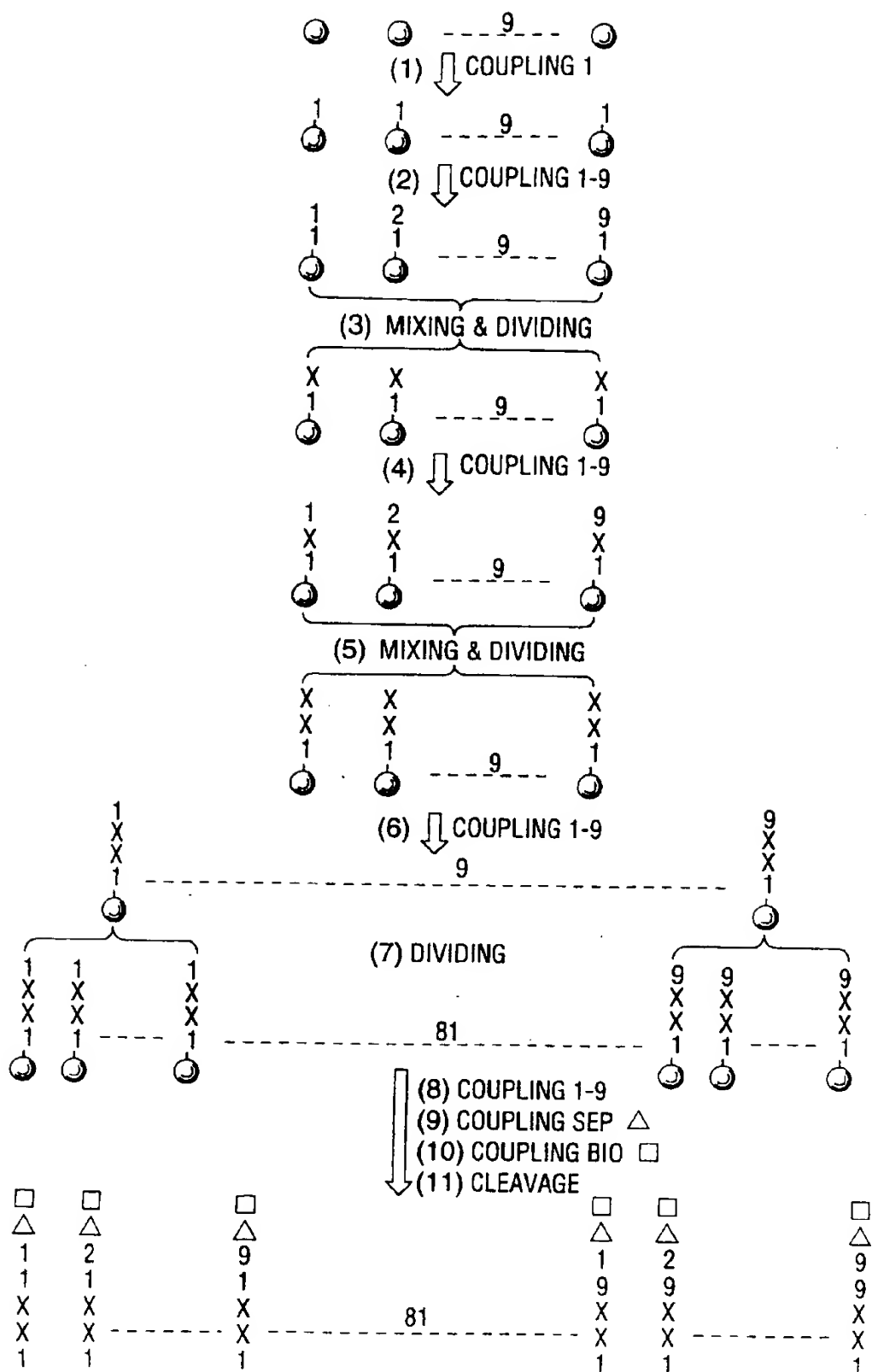


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16435

A. CLASSIFICATION OF SUBJECT MATTER																				
IPC(6) : G01N 33/53 US CL : 435/7.1, 7.25 According to International Patent Classification (IPC) or to both national classification and IPC																				
B. FIELDS SEARCHED																				
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/7.1, 7.25																				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, Medline, CAS. search terms: affinity labeling, library, binding, peptide library																				
C. DOCUMENTS CONSIDERED TO BE RELEVANT																				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
Y	BRUMMEL, C. L. et al. A mass spectrometric solution to the address problem of combinatorial libraries. Science.15 April 1994, Vol. 264, pages 399-401, see entire document.	11,12																		
Y	GORDON, E. M. et al. Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions. J Med.Chem.13 May 1994, Vol 37, No. 10, pages 1386-1401, see entire document.	7,8,15,16, 17																		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																				
<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier document published on or after the international filing date</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"A"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A"	document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means			"P" document published prior to the international filing date but later than the priority date claimed		
* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																		
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																		
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																		
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A"	document member of the same patent family																		
"O" document referring to an oral disclosure, use, exhibition or other means																				
"P" document published prior to the international filing date but later than the priority date claimed																				
Date of the actual completion of the international search 13 JANUARY 1998		Date of mailing of the international search report 25 FEB 1998																		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer NEAL A. MUSTO, PH.D. Telephone No. (703) 308-0196																		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16435

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KERR, J. M. et al. Encoded combinatorial peptide libraries containing non-natural amino acids. J.Am.Chem.Soc. 1993, Vol 115, pages 2529-2531, see entire document.	1-3, 6
Y	SORENSEN, P. et al Identification of the interleukin-3 receptor using an iodinated, cleavable, photoreactive cross-linking agent. J. Biol. Chem. 15 July 1986, Vol. 261, pages 9094-9097, see entire document.	1-3, 6 11,12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/16435

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 6-8, 11, 12, 15-17

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

